Dengue viruses (DEN) belong to the family *Flaviviridae* and consist of 4 distinct serotypes (DEN-1–4). All 4 DEN serotypes cause disease and are estimated to infect up to 100 million people annually. Disease manifestations range from mild, self-limited, acute, dengue fever to the more severe, life-threatening dengue hemorrhagic fever and dengue shock syndrome.1 After a first infection with any of the 4 DEN serotypes, long-term, homologous, protective immunity is thought to occur. Shorter term, cross-protective immunity to other serotypes also has been demonstrated, but such immunity wanes after a few months.2 In fact, infection with a second serotype of dengue virus has been strongly associated with dengue hemorrhagic fever and dengue shock syndrome. Infections with a third serotype also have been occasionally documented. The occurrence of secondary and tertiary dengue infections, and particularly the association of secondary infections with dengue hemorrhagic fever–dengue shock syndrome, have led to the conclusion that an effective dengue vaccine should induce long-term protective immunity against all 4 serotypes of virus.

In spite of several decades of research, there is no approved dengue vaccine for human use. Most research efforts have focused on the development of live attenuated vaccines selected by serial passage in mice or mammalian cell cultures and recombinant protein vaccines.3–11 Efforts to develop genetically modified and chimeric live attenuated vaccines or DNA vaccines also have emerged.12–16 In most dengue vaccine efforts, the long-term goal has been to develop a tetravalent vaccine that would induce neutralizing antibody against all 4 viral serotypes. Neutralizing antibody is thought to be an important component of protective immunity on the basis of studies of infants born to dengue-immune mothers as well as on the basis of passive antibody transfer studies in animals.17,18 However, to date, only a few studies have been published that discuss the use of bivalent vaccines, and to our knowledge, no detailed studies about trivalent or tetravalent dengue vaccines have been published.

We previously demonstrated that a recombinant fusion protein containing amino acids (aa) 298–400 of the B domain of the DEN-2 envelope (E) protein fused to the maltose binding protein (MBP) of *Escherichia coli* was immunogenic in mice, induced anti-DEN neutralizing antibody as compared with mice immunized with MBP alone, and conferred protection in 80% of mice after intracerebral challenge.11 We have now constructed individual recombinant fusion proteins containing the B domains of DEN-1 (Western Pacific, aa 293–412), DEN-3 (H87, aa 297–398), and DEN-4 (Dominican Republic 1988, aa 298–400) fused to MBP. In the present study, we evaluated the ability of these constructs administered to mice as a tetravalent vaccine to induce antibody against all 4 serotypes of dengue virus.

Separate groups of 6 to 10 BALB/c mice were inoculated intramuscularly on days 0, 15, and 29 with 50 μg of one of the DEN-MBP fusion proteins (monovalent vaccination) or an equal mixture of all 4 DEN-MBP fusion proteins (tetravalent vaccination), adsorbed on 0.1% alum. Blood was drawn from the retro-orbital sinus on day 56 after the first inoculation, and pooled sera were assayed for DEN antibodies by enzyme-linked immunosorbent assay (ELISA) and plaque reduction neutralization test by means of LLC-MK2 cells.11,19 The virus strains used to prepare antigens for the ELISA and for performing the neutralization tests were DEN-1 (Western Pacific), DEN-2 (New Guinea C), DEN-3 (CH53489), and DEN-4 (341750).

Mice inoculated with the monovalent DEN-MBP subunit vaccines developed a strong IgG antibody response against the homologous purified whole virus used as an antigen in the ELISA (Table 1). Homologous endpoint titers ranged from 1:12,800 to 1:3,200. Cross-reactive antibody titers were at least 4-fold lower than the homolo-

### Table 1

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Reciprocal end-point titers by dengue virus serotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEN-1</td>
</tr>
<tr>
<td>DEN-1</td>
<td>6,400</td>
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<tr>
<td>DEN-2</td>
<td>&lt;100</td>
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<tr>
<td>DEN-3</td>
<td>&lt;100</td>
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<tr>
<td>DEN-4</td>
<td>&lt;100</td>
</tr>
<tr>
<td>DEN-1–4</td>
<td>12,800</td>
</tr>
</tbody>
</table>

*The cutoff value for seropositivity was set at 20 because the mean adjusted optical density + 3 standard deviations for negative control sera was consistently below this value.

Purified whole dengue virus was used as an antigen.
after immunization of mice with live dengue virus. The DEN1–4 tetravalent titer was 8-fold lower than the monovalent titer. However, because neither the DEN-1, DEN-2, or DEN-3 monovalent vaccine groups developed cross-reactive DEN-4 antibody, the DEN-4 antibody seen with the tetravalent vaccine probably represents a serotype-specific response to the DEN-4 recombinant component.

All of the monovalent vaccine groups of mice developed DEN neutralizing antibody (Table 2). The mice that received DEN-2–MBP and the mice that received DEN-3–MBP developed high homologous titers and no detectable cross-reactive neutralizing antibody. The DEN-1–MBP and the DEN-4–MBP groups developed cross-reactive neutralizing antibody against DEN-3 and DEN-2, respectively, but the titers were ~3-fold lower than the homologous titers. The mice that received the tetravalent vaccine developed neutralizing antibody to all 4 dengue virus serotypes. Neutralizing antibody titers were somewhat lower (~2-fold) than the monovalent homologous titers, but in all cases, they were much higher (~6-fold) than the monovalent cross-reactive neutralizing antibody titers.

A previous report showed that baculovirus-expressed recombinant DEN-2 and DEN-3 truncated envelope protein inoculated concomitantly in mice-induced neutralizing antibody to both serotypes of dengue virus.17 Another report that used a baculovirus-expressed recombinant hybrid DEN-2 and DEN-3 envelope protein also showed that immunized mice produced antibody to both DEN-2 and DEN-3 virus by Western blot assay.19 However, our study is the first to show that a dengue tetravalent recombinant protein vaccine can induce a strong neutralizing antibody response to all 4 dengue virus serotypes. Furthermore, the titer of neutralizing antibody induced against each dengue virus serotype after immunization with the tetravalent recombinant vaccine is as high or higher than titers reported after immunization of mice with live dengue virus.3,20 These encouraging results suggest that further investigation of the DEN-MBP tetravalent recombinant subunit vaccine is warranted. Studies of nonhuman primates are planned to assess the ability of this vaccine to prevent viremia after challenge with live virus and to determine the duration of protection.

The animal-use research protocol in this study was reviewed and approved by the Naval Medical Research Center’s Institutional Animal Care and Use Committee according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animals, NIH publication 92–3415.

Financial support: This research was supported by Naval Medical Research and Development Command work unit 62787A.870.S.1442.

Disclaimer: The opinions and assertions herein are those of the authors and are not to be construed as official or as reflecting the views of the U.S. Navy or the naval service at large.

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REFERENCES


TABLE 2

Neutralizing antibody in mouse sera after immunization with dengue recombinant protein vaccines

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>DEN-1</th>
<th>DEN-2</th>
<th>DEN-3</th>
<th>DEN-4</th>
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<tbody>
<tr>
<td>DEN-1 (n = 6)</td>
<td>240</td>
<td>&lt;20</td>
<td>70</td>
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<tr>
<td>DEN-2 (n = 10)</td>
<td>&lt;20</td>
<td>1024</td>
<td>&lt;20</td>
<td>&lt;20</td>
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<tr>
<td>DEN-3 (n = 6)</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>510</td>
<td>&lt;20</td>
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<tr>
<td>DEN-4 (n = 6)</td>
<td>&lt;20</td>
<td>25</td>
<td>&lt;20</td>
<td>80</td>
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<tr>
<td>Tetravalent (n = 10)</td>
<td>100</td>
<td>450</td>
<td>480</td>
<td>35</td>
</tr>
</tbody>
</table>

* Mice were immunized with one of four monovalent vaccines or a combination of all four vaccines. Sera were from day 56 after first inoculation. Results are expressed as the reciprocal of 50% plaque reduction neutralization titer against each dengue virus serotype.